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# Limited Dynamic Range of Immune Response Gene Expression Observed in Healthy Blood Donors Using RT- PCR

Title for running head: Gene Expression in Healthy Subjects

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**Abstract**

The use of quantitative gene expression analysis for the diagnosis, prognosis, and monitoring of disease requires the ability to distinguish pathophysiological changes from natural variations. To characterize these variations in apparently healthy subjects, quantitative real-time PCR was used to measure various immune response genes in whole blood collected from blood bank donors. In a single-time-point study of 131 donors, with 48 target genes, 43 were consistently expressed and, 34 followed approximately log-normal distribution. Most transcripts showed a limited dynamic range of expression across subjects. Specifically, 36 genes had standard deviations (SD) of 0.44 to 0.79 cycle threshold ($C_T$ ) units, corresponding to less than a 3-fold variation in expression. Separately, a longitudinal study of 8 healthy individuals demonstrated a total dynamic range (> 2 standard error units) of 2- to 4-fold in most genes. In contrast, a study of whole blood gene expression in 6 volunteers injected with LPS showed 15 genes changing in expression 10- to 90-fold within 2 to 5 hours and returning to within normal range within 21 hours. This work demonstrates: (1) the dynamic range of expression of many immune response genes is limited among healthy subjects; (2) expression levels for most genes analyzed are approximately log-normally distributed; and (3) individuals exposed to an infusion of bacterial endotoxin, lipopolysccharide (LPS), show gene expression profiles that can be readily distinguished from those of a healthy population. These results suggest that normal reference ranges can be established for gene expression assays, providing critical standards for the diagnosis and management of disease.

## Introduction

Recent developments in gene and protein expression analysis technology have suggested that gene expression is a key indicator of an individual's pathophysiologic status (1, 2, 3, 4). Consequently, clinical application of gene expression technology will vastly improve upon the current approaches for monitoring health and disease. Compelling associations between gene expression and disease have been demonstrated in many studies ranging from inflammatory disease to cancer. For instance, studies have pointed to abnormal gene expression in peripheral blood mononuclear cells in lupus patients compared to healthy controls (5,6). Other studies have found differences in gene expression patterns between cancerous liver or pancreatic tissue and non-tumor liver and pancreatic tissues (7,8). Additionally, gene expression profiling of breast tumor biopsy tissue correlated with therapeutic response to treatment (9). Results from these studies demonstrate that measurements of gene expression can be used in the diagnosis and monitoring of disease. However, a key requirement for clinical application of gene expression technology is distinguishing between natural variations in gene expression among healthy subjects and changes associated with a disease condition. The establishment of a normal range of expression for a particular population is required as a "reference range" (10).

Immune function is controlled by a network of molecular and cellular pathways. It is well recognized that suppressed immune responses (e.g., immunosuppressive therapies and AIDS) or excessive responses (e.g., acute respiratory distress syndrome and autoimmunity) can contribute to disease. Thus, homeostatic control and tight regulation of responses are fundamental characteristics of the immune system. For example, in the absence of disease, body temperature remains relatively constant within an

individual, suggesting that the body strives to hold its temperature close to a defended set point. During a response to infection, the inflammatory cytokines interleukin 1, interleukin 6, and tumor necrosis factor are released into the blood and bind with receptors in the hypothalamus resulting in fever (11). However, immune cells also manufacture and release factors, such as interleukin 1 receptor antagonist and interleukin 10, that counteract the effects of pro-inflammatory cytokines and reduce body temperature (12,13). As a result, body temperature rises only moderately during many fever episodes, and returns to its previous set point upon clearance of the infection. This and other evidence (14) imply that inflammatory/immune genes may be tightly regulated. It is further hypothesized that immune system homeostasis would be reflected in a narrow range of expression levels or set points for key molecules in these pathways among healthy subjects.

In certain gene expression studies, reproducible patterns in subsets of genes have been noted in normal tissues (15-18). The majority of these studies have used microarrays to explore the patterns of expression in isolated blood cell fractions (15, 18) or other target tissues, including retina (16) and skin (17). Some studies (16, 19) have used replicate arrays to assess the relative contributions of technical and biological factors to the overall variation in measurement values. The results show inter-individual variation for gene expression, as well as variation over time within an individual. In addition, gene expression can be sensitive to sources of technical variability, such as time after phlebotomy and method of RNA isolation (20-23). Even within a platform, such as microarrays, considerable divergence is reported across various platforms (24).

In recent years, quantitative real-time (QRT) PCR has emerged as an effective and reproducible tool for transcript analysis (25). It measures relative abundances through PCR-based synthesis of target gene amplicons and activation of target specific

fluorescent probes. The amount of fluorescence generated during the exponential amplification phase provides robust comparative abundance measurements for different amplicons in the same or different wells (25). Whole blood contains representative populations of all the mature cells of the immune system as well as secretory proteins associated with cellular communications (26). The earliest observable changes of cellular immune activity are altered levels of gene expression within the various immune cell types (27). Therefore, quantitative RT-PCR (QRT-PCR) can be a very effective technology for reproducibly quantifying gene expression in whole blood.

In studies reported here, we explored the variation among apparently healthy blood bank donors in the expression of a set of genes involved in immune responses. QRT-PCR was used to measure immune-related gene expression in whole blood samples, using procedures designed to sustain a high level of precision (repeatability and reproducibility). We tested the observed distribution of values to determine if it was consistent with sampling from a log-normal distribution, as has been asserted for many genes (28, 29), and computed maximum likelihood estimates for the parameters of this distribution. We used statistical models to estimate the contributions of gender, age, and ethnicity to the overall differences in expression among subjects. By performing replicate measurements on longitudinal samples from a group of 8 donors, we computed relative proportions of variance arising from technical, temporal, and inter-subject variability. Finally, to obtain limits for the dynamic range of expression achievable with a strong inflammatory stimulus, we performed time course measurements for several immune response genes in a group of healthy volunteers challenged with an infusion of the bacterial endotoxin, lipopolysaccharide (LPS).

## Materials and Methods

### Donor Selection

Single time point blood samples from 131 blood donors satisfying American Red Cross blood bank standards (30) were obtained from three individual donor centers operated by Bonfils Blood Center, Denver, CO, USA. The samples were drawn on three different days over a three month period. Subject ages ranged from 22 to 69 years, with a median age of 44 years; age was not recorded for 61 subjects. Females (N=64) and males (N=67) were represented in about equal numbers. Ethnicity was reported as white/non-Hispanic for 109 subjects, as Hispanic for 19, as African-American for 2, and Asian/Pacific Islander for 1. No subjects in this study showed overt signs of disease that would make them ineligible to donate blood under American Red Cross standards. However, because we cannot rule out undetected disease in the subjects, we refer to them as apparently healthy (18).

In addition, longitudinal samples were drawn from 8 volunteers (3 female, 5 male, age range 23-50 years) from the Denver area. Samples were collected from these donors approximately once per month for six to eight months, yielding a total of 58 samples.

Samples from the blood donor subjects were collected under Western Institutional Review Board Study Number 20010324. The studies were also reviewed by the Lawrence Livermore National Laboratory Institutional Review Board. Written informed consent was obtained from all volunteers.

In a separate study, six healthy volunteers were injected intravenously over 1 minute with a single dose (30 units/kg) of Gram-negative bacterial lipopolysaccharide (LPS), according to an approved protocol at Guys Hospital, London, UK. Blood samples were drawn and assayed before the LPS injection (0h), 2h and 5h after LPS injection. Three of six subjects were additionally drawn and assayed 21h after LPS injection. Subjects

were adult male volunteers that signed an informed consent form. Medical history, physical examination, routine laboratory examination and electrocardiogram were all normal. Subjects did not use any medication or have any significant illness within 8-weeks of the study.

**Sample handling, purification of RNA and preparation of cDNA**

Blood was collected from study subjects by standard phlebotomy methods using a 21-gauge butterfly needle and PAXgene™ Blood RNA Tubes (QIAGEN, no. 762115, Valencia, CA) to stabilize messenger RNA (mRNA) against degradation and prevent induction of new mRNA expression (23). Samples were gently mixed by inversion, and sat at room temperature for 2-24 h to ensure complete nucleic acid stabilization. Samples were then frozen at –70°C, and batch shipped on dry ice in compliance with IATA shipping regulations.

Total RNA from PAXgene™ Blood RNA samples was extracted within 30 days of collection using the PAXgene™ Blood RNA Kit (QIAGEN, no. 762134). RNA samples were treated with RNase-Free DNase I (QIAGEN, no. 79254) for digestion of contaminating genomic DNA, using manufacturer recommended protocols during the purification process. Purified RNA samples were placed at –80°C for long term storage.

First strand cDNA was synthesized with random hexamer primers, using TaqMan® Reverse Transcription reagents (Applied Biosystems, no. N808-0234). Approximately 250 ng of RNA was added to a prepared reverse transcription reagent mixture consisting of PCR Buffer II, 1X; $MgCl_2$, 5.5 mM; random hexamers, 2.5 µM; dNTP blend, 2 mM; RNase Inhibitor, 40 U; and MultiScribe™ Reverse Transcriptase, 125U. Samples were incubated at ambient temperature for 10 minutes with subsequent incubation at 37°C for 60 minutes. Following the 37°C incubation, samples were incubated at 90°C for 10 minutes and immediately chilled on ice. Newly synthesized cDNA samples were then

placed at –80°C for storage. Each cDNA sample was quality control tested for RNA quantity and quality prior to QRT-PCR analysis of target genes using quantitative PCR analysis (QPCR; ABI Prism® 7700 Sequence Detection System, Applied Biosystems, Foster City, CA) of the 18S rRNA and β-actin.

**QRT-PCR analysis of target genes**

Primer/probe reagents were custom designed to achieve three performance criteria: 1) single gene specificity of amplification as tested by gel electrophoresis; 2) dilutional linearity of amplification performance over 2 orders of magnitude; and 3) optimal amplification efficiency of 100±6%, to yield a two-fold change in transcript per $C_T$ unit (31). Primer/probe sets were designed to span 90-120 base pairs, optimized for robust amplification and specificity, minimization of secondary hybridization, and consistent performance. Quality control testing of reagents and manufactured plates ensure that amplification specificity and efficiency remain within established metrics during storage and new synthesis of nucleotides.

Amplification specificity was tested by QRT-PCR with a custom cDNA standard template of induced whole blood and cell lines. Specificity was determined by the size, number, and DNA sequence of the amplified product. The size and number of amplified products was determined by agarose gel electrophoresis. Amplified products were electrophoresed on a 4% agarose gel to visualize the number of DNA bands present. The molecular weight of each band was determined by comparison to known molecular weight markers (Fisher Scientific, no. PR-G1741, Hampton, NH). The presence of a single DNA band of the correct size suggested specific amplification of the intended gene sequence. In certain cases, the amplified product DNA sequence was compared to the published sequence. Primer/probe amplification of genomic DNA was investigated using purified genomic DNA rather than cDNA as the template for QRT-

PCR. The formation of primer dimers and spurious amplification was also investigated using DEPC water as a "no template" control for the QRT-PCR assay.

Amplification efficiency of a primer/probe set was determined by a dilutional linearity assay, using 5 serial dilutions of the standard cDNA template and running PCR reactions on each dilution in replicates of 4. Two or more versions of each target gene primer/probe set were designed and tested to select for both amplification efficiency and specificity. Similarly, each new primer/probe reagent lot was monitored to ensure matched amplification specificity and efficiency to previous primer/probe reagent lots.

Target gene transcripts were analyzed by QRT-PCR for each cDNA preparation using 2X TaqMan® Universal PCR Master Mix (Applied Biosystems, #4305719, Foster City, CA) and Source MDx's proprietary primer-probe sets. Reactions were run in sets of four replicates per gene (24 gene targets in a 96-well plate) on an ABI Prism® 7700 Sequence Detection System. Each well also contained the specific primers and probe set to measure 18S rRNA as an internal control. The amount of cDNA template added to each reaction was held to a relatively narrow range, as determined by the cDNA quality control measurement of 18S RNA.

**Data analysis**

The difference between the fluorescence $C_T$ for the target gene and the endogenous control (18S rRNA) is presented as a $\Delta C_T$ value [$C_T$(target) – $C_T$(control)]. For reference, a $\Delta C_T$ of 2 is approximately equivalent to a 4-fold change in the amount of the transcript. For example, at baseline, TGFB may have a $\Delta C_T$ value of 16, after treatment, that $\Delta C_T$ value may increase to 18. This change represents a 2 $\Delta C_T$ difference or a decrease of 75% (1/4). The $C_T$ reporting system and estimation of relative gene expression is well described in the literature (32).

$C_T$ values above 37 were not used in the analysis, because they correspond to gene expression levels below the linear range of the assay. Values over this threshold were obtained for varying proportions of samples, depending on the gene and the study population examined. For the single-time-point samples, the mean and SD of the underlying $\Delta C_T$ distribution were inferred by maximum likelihood estimation (MLE), under the assumption of a normal distribution, for genes having up to 50% of their $C_T$ values over the threshold. Distribution parameters and dynamic ranges were not computed for genes with more than 50% of $C_T$ values greater than 37.

**Tests for normality**

Since $\Delta C_T$ values are roughly proportional to the logarithm of the corresponding mRNA abundances, we used a combination of analytical methods to test $\Delta C_T$ values for each gene for departures from normality.

The Anderson-Darling and Shapiro-Wilk tests were used to test the data against the null hypothesis that the observed values were sampled from a normal distribution, parameterized by the observed mean and standard error. These tests differ in their sensitivity to outliers and in the weight given to central versus outlying values. Smaller p-values from these tests indicate rejection of the null hypothesis, i.e., deviation from normality.

We also generated plots of the quantiles of each gene's $\Delta C_T$ values against the corresponding quantiles of a standard normal distribution (Q-Q normal plots), together with histograms and normal density curves, in order to graphically characterize their deviations from normality.

**Linear mixed effect model analysis**

Previous reports on longitudinal gene expression data sets (16, 19) suggest that, for many genes, expression levels in repeated samples from the same subject are relatively stable compared to inter-individual differences, even when the repeat samples are separated by time periods of several weeks. To quantify the relative magnitudes of inter-subject versus temporal and technical variability in apparently healthy, untreated subjects, we fitted a linear mixed-effects (LME) model to the longitudinal study data. In this data set, each $\Delta C_T$ measurement was associated with a gene *g*, subject *i*, sample index *j*, and replicate *k*. An LME model for these data is described by equation 1:

$$(\Delta C_T)_{gijk} = \alpha_g + u_{gi} + \beta_{gj} + v_{gij} + \varepsilon_{gijk}$$

where $\alpha_g$ is an intercept term dependent on the gene only, $u_{gi}$ is a random effect due to inter-subject variability, $\beta_{gj}$ is a fixed effect due to systematic variations in processing affecting all samples drawn at the same time point, $v_{gij}$ is a random effect representing variability among samples from the same subject, and $\varepsilon_{gijk}$ is an error term encompassing all residual sources of variability between replicates. The random effects $u_{gi}$ , $v_{gij}$ and $\varepsilon_{gijk}$ are assumed to be normally distributed with mean zero and variances $\sigma^2_S$, $\sigma^2_{T,}$ and $\sigma^2_{R,}$ respectively. A restricted maximum likelihood (REML) algorithm (33) was used to fit the model parameters $\alpha_g$, $\beta_{gj}$, $\sigma^2_S$, $\sigma^2_T$, and $\sigma^2_R$ to the data.

In addition, it is useful to quantify the contributions to inter-subject variability arising from subject characteristics such as gender, age, and ethnicity. All three of these parameters were recorded for 68 subjects in the single-time-point study. Expression data for these subjects was fitted to the LME model described by equation 2:

$$(\Delta C_T)_{gik} = \alpha_g + \beta_g(G_i, E_i) + \zeta_g(G_i, E_i)A_i + u_{gi} + \varepsilon_{gik}$$

where $\alpha_g$ is an intercept term dependent on the gene only, $G_i$, $A_i$ , and $E_i$ are the gender, age, and ethnicity of subject *i*, $\beta_g(G,E)$ is a gene-specific offset for the given gender and ethnicity, $\zeta_g(G,E)$ is the slope of a linear age effect depending on both gender and ethnicity, $u_{gi}$ is a random effect due to inter-subject variability not explained by age, gender, or ethnicity, and $\varepsilon_{gik}$ is an error term encompassing all residual sources of variability between replicate PCR reactions for a given sample. After fitting this model, the percentage contribution of gender, age, and ethnicity effects to the inter-subject variance for gene *g* was estimated by equation 3:

$$(PC)_g = 100 \,/\, (1 + \sigma^2_S \,/ \sum_{ik} (((\text{predicted } \Delta C_T)_{gik} - (\text{mean } \Delta C_T)_g)^2)/(N-1))$$

where *N* is the total number of measurements for gene g, $\sigma^2_S$ is the variance parameter estimated for the distribution of the random subject effects, "predicted $\Delta C_T$" is the value predicted from the fixed effects portion of equation 2, and the mean $\Delta C_T$ is computed over all measurements for gene g.

All data analyses were performed using the R open source programming environment for statistical computation (34). LME models were programmed using the R package "nlme" (33).

## Results

### Most genes exhibit limited dynamic range of expression across subjects in single-time-point measurements

A series of studies were undertaken to examine the expression of immune-related gene transcripts in whole blood of apparently healthy subjects. In the largest single-time-point study, blood was collected from 131 blood donors following the American Red Cross donor standards and analyzed for the expression of 48 inflammation- and immune-related gene transcripts. These transcripts encode cell surface molecules, such as CD4, CD14, CD19, and ICAM-1; signaling molecules, such as PTGS2 (COX2), PLA2G7, and NFkB; cytokines, such as IL1B and TGFB; proteinases, such as ELA2; and proteinase inhibitors (see Table 1). The overall range of $C_T$ values for the 48 genes studied is plotted in Figure 1. The bars in the plot encompass the central 90% of the observed values (i.e., they extend from the $5^{th}$ to the $95^{th}$ percentiles), whereas the "whiskers" on either end of the bar extend to the extreme values. For genes with expression levels sampled from a log-normal distribution, the ends of the bars would correspond to 1.64 SD on either side of the mean $C_T$.

Of the 48 genes profiled in this study, two important signals of inflammation, IL6 and CXCL2, lacked detectable expression in a majority of the apparently healthy subjects, and their $C_T$ values were at or greater than 37. Dynamic ranges and variance components were not computed for these genes. For the remaining 46 genes, the estimated SD of the $\Delta C_T$ values ranged from 0.44 to 1.46, and was below 0.792 for 36 of the 46 genes, as shown in Table 1. Thus, the dynamic range of expression extending 2 SD in either direction from the geometric mean was less than $2^{2 * 0.792}$ or 3 fold change (32). For normally distributed $\Delta C_T$ values, this range covers 95.4% of the sample measurements. The distribution of dynamic ranges corresponding to a $\pm$ 2 SD span is

shown in Figure 2. The highest dynamic range observed was 7.53 fold change units for IL8. The SDs of $\Delta C_T$ values were independent of the mean $\Delta C_T$, indicating that the dynamic ranges did not depend on a gene's expression level.

**The majority of genes have expression values following log-normal distributions**

Commonly used parametric tests for differential gene expression between groups of samples, such as t-tests and analysis of variance, are based partly on the assumption that the values being compared are sampled from normal distributions. Although it is commonly asserted that transcription levels of many genes are log-normally distributed (28, 29), it is important to test this assumption in order to use such tests for disease diagnosis and detection. The majority of expressed transcripts followed approximately log-normal distributions, according to the Anderson-Darling and Shapiro-Wilk tests (Table 1, Figure 3). The gene most closely following a normal distribution of $\Delta C_T$ values was IL1R1 (Figure 3A), with an Anderson-Darling p-value of 0.945. Among the 46 genes tested, 34 had p-values greater than 0.001. All genes had unimodal distributions; the deviations from normality involved moderate degrees of left or right skewness, and/or heavy or light tails. Although these departures were not dramatic, they will need to be incorporated into the predicted error rates for diagnostic tests based on expression of these genes.

Of the 48 genes shown in Table I, the gene deviating most from a normal distribution of $\Delta C_T$ values was TNFSF5 (CD40 ligand, Figure 3B), with an Anderson-Darling p-value of $1.52 \times 10^{-10}$. The observed distribution is characterized by a heavy tail and large $\Delta C_T$, suggesting the presence of a subpopulation with an unusually low expression level of this gene.

**Minor variations in expression may be based on gender, ethnicity, and age.**

Table 2 shows the contributions of gender, age, and ethnicity on inter-individual variation estimated by the LME model (equation 2). For the 43 genes examined, the observed effects of gender, ethnicity, and age were small. Only 10 genes had contributions from these effects, explaining more than 20% of the inter-subject variance; the maximum contribution was only 27.9% for NFKB1. For most genes, gender effects accounted for most of this contribution. Fifteen genes showed significant gender differences (unadjusted p-value < 0.05), but the largest fold change from females to males was only 1.62 for TNFSF6. Likewise, only moderate ethnicity effects were observed. Five genes (MPO, MYC, TNFSF6, ELA2, and HMGB1) showed significant differential expression between white (non-Hispanic) and Hispanic subjects, with the largest change being a 2.5-fold over-expression of ELA2 in Hispanic females relative to white females.

Age effects were difficult to measure in this data set, due to the markedly different age distributions between the female and male blood donors. Male blood donors had a median age of 53 years, compared to 43 years for females. Therefore, gender and age effects are potentially confounded. The LME model defined in equation 2 addresses the confounding factors by fitting the $\Delta C_T$ versus age data to different slopes for each gender/ethnicity combination. According to the LME model, three genes (IL18, ELA2, and C1QA) had significant age effects for at least one gender/ethnicity combination. For all three of these genes, the fitted slopes were markedly different between genders. For example, age had virtually no effect on IL18 expression in white males, while in white females the slope corresponded to a 2-fold increase from age 23 to age 69. Similarly, the fitted slopes suggest dramatic differences in age effects among ethnicities. Overall, the size of the sample is too small to reliably estimate ethnic differences.

**Variation of expression within subjects over time is limited**

To compare the contributions of inter-subject, temporal, and technical components to the overall variation in gene expression, we fit the LME model (equation 1) to the longitudinal set of measurements described in *Materials and Methods*. For this data set, we fit the model for each of 29 genes with detectable expression in at least 90% of the samples to obtain, for each gene, a set of variance parameters $\sigma^2_S$ , $\sigma^2_{T,}$ and $\sigma^2_R$. These are approximate estimates of the contributions to the total variance from inter-subject variation, variation among samples taken at different times from each subject, and residual variation between replicate reactions, respectively.

The results of the initial LME model analysis are summarized in Figure 4, which shows the fitted standard error parameters $\sigma^2_S$ , $\sigma^2_{T,}$ and $\sigma^2_R$ for each gene. For 6 of the 29 genes examined (CD19, TNFSF13B, HMOX1, C1QA, CD8A, and CD4), inter-subject variation comprised more than 50% of the total variance of $\Delta C_T$ values. For the remaining 23 genes, variation between samples taken at different times was the largest component. However, the magnitude of the temporal variation was limited; the parameter $\sigma_T$ ranged from 0.36 $\Delta C_T$ units for the gene PTPRC to 0.72 $\Delta C_T$ units for MMP9. The dynamic ranges corresponding to $2\sigma_T$ ranged from 1.66 to 2.72 fold change units. Since measurements from samples taken over a period of 8 months may be subject to several sources of technical variation (e.g., instrument calibration, reagent lots, and variations in sample handling), these ranges can be considered upper limits on the true temporal variation of expression for the genes analyzed.

**LPS stimulation induces transient gene expression changes in excess of natural variation**

To demonstrate that changes marked beyond the normal reference range occur, gene expression was measured in blood collected from healthy subjects injected with LPS. Healthy subjects who receive an injection of LPS experience mild fever and flu-like

symptoms that subside within 24 hours (35). The expression of a subset of genes with significant changes at any time point after LPS injection, are shown in Figure 5. Reference ranges (mean $\pm$ 2 SD) for healthy subjects are indicated by dashed lines. The plotted $\Delta\Delta C_T$ values are computed relative to the mean $\Delta C_T$ for the apparently healthy blood donors. Individual time courses are shown for each subject. Twenty-seven genes had significant changes in expression in LPS-injected subjects at any time post-infusion relative to apparently healthy blood donors, with adjusted false discovery rates of less than 5%. Each of these genes had pre-injection expression levels within the normal reference range for apparently healthy blood donors; showed increased or decreased expression at 2 and/or 5 hours post-infusion; and most returned to the normal expression range by 21 hours after infusion. Fifteen genes increased or decreased expression by a factor greater than 10-fold, and two (MMP9 and IL1RN) increased more than 90-fold (Figure 5). Since the innate immune system's immediate response to LPS infusion is the production of inflammatory mediators by monocytes, it is not surprising that the genes showing substantial increases in expression include cytokines and chemokines associated with the monocyte/macrophage lineage, such as TNF, IL1B, CXCL1, and IL18. Key cell surface markers (ICAM1, CD14) and signaling molecules (PTGS2 / COX-2) also respond. Interestingly, the anti-inflammatory regulator IL1RN, which blocks the binding of IL1 to its receptor, was one of the two most "over-expressed" genes. This fits with the premise that inflammatory processes are tightly regulated by coordinated expression of pro-inflammatory and anti-inflammatory factors. These include genes with significant decreases in expression such as PLA2G7 and TNFSF5 (CD40 ligand) (see figure 5).

## Discussion

The studies reported here are an initial step toward establishing “normal reference ranges” for the expression of genes related to inflammation and immunity. Several key observations emerged: First, the dynamic range of expression of most immune response genes is relatively limited among apparently healthy subjects. Second, expression levels for most genes analyzed are approximately log-normally distributed. Third, individuals exposed to bacterial endotoxin have gene expression profiles that are easily (albeit transiently) distinguished from those of an apparently healthy population. In developing the methods for these studies, it was also observed that multiple technical factors, including sample handling procedures, PCR reagents, and instrument calibration, contribute to the overall variation, which must be carefully controlled. Taken together, these observations support both the usefulness and practicality of establishing normal reference ranges for gene expression assays related to immune system function.

A variety of biological factors may contribute to the variation of expression observed in apparently healthy subjects (18). In general, these factors can be divided into intrinsic (e.g., age, gender, genetics) and extrinsic factors (e.g., inflammatory, autoimmune disease, cancer, infections, and metabolism). The apparently healthy blood donor population studied here may have included individuals with sub-acute illnesses or chronic conditions that contributed to the variability in expression of some immune response genes. Many chronic inflammatory and atopic diseases, such as arthritis, asthma, ulcers, gastritis, and allergies are highly prevalent in the US adult population, with frequencies ranging from 7% to 27% (36). Nonetheless, individuals with these conditions are deemed “healthy” and permitted to donate blood, provided these “chronic conditions are bring treated and the condition is under control, and they “feel well and are able to perform normal activities” (30).

Atherosclerosis is another highly prevalent condition, which develops over several years and is asymptomatic in its early or even late stages. Several studies have demonstrated an elevation of C-reactive protein and other markers of inflammation in early stages of cardiovascular disease (37, 38). Chronic infections with viruses (cytomegalovirus, Epstein-Barr virus, genital herpes, and human papillomavirus), bacteria (*Helicobacter pylori*) and protozoans (*Toxoplasma gondii* ) also are common in the US population, but do not consistently produce symptoms in immunocompetent persons. Periodic reactivation and suppression of these infections may account for some of the background variation in immune response gene expression. Dietary influences on immune system gene expression may include consumption of omega-3 fatty acids, arginine, and other nutrients as well as vegetarian diets (39, 40).

Age, gender, and ethnicity also may contribute to the inter-subject variation observed for several transcripts. However, the contributions of these factors appeared to be modest in the present study. Variations associated with age and gender, have been previously reported (18, 41, 42) with some gender differences being directly attributable to differences in sex chromosomes (18). Several studies (18, 42) have observed individual differences in interferon responsive genes among individuals suggesting further stratification in an apparently normal healthy subject group. Larger studies specifically targeting some of these factors are needed to elucidate the effects so that populations can be stratified for more precise diagnostic resolution.

Intrinsic and extrinsic factors can also alter the proportions of blood cell types such as neutrophils, monocytes, and lymphocytes, as well as the relative expression of individual transcripts within each cell type. These effects combine to produce the observed variation in transcript abundances in whole blood. The individual contributions of cell

populations and gene regulation within cell types could be examined using flow cytometry combined with QRT-PCR, and deserve further study.

Given the variety of factors that can affect the expression of immune response genes in a blood donor population, it is remarkable that the overall dynamic range of expression is not wider than observed in the present study, whereas larger, up to 90-fold, but transient changes can be induced by the severe acute inflammatory stimulus, LPS. In other disease studies, such as rheumatoid arthritis and lupus differences in gene expression from apparently healthy normals are more modest, 2 to 5 fold (43). These observations support the view that expression of these genes is maintained within limits by regulatory mechanisms, possibly to reduce the danger of tissue damage from constant activation of immune responses, while allowing appropriate responses to infectious threats. The limited dynamic range observed supports the development of expression-based diagnostics, allowing expression outside the normal reference range to indicate the presence of infections, cancer or indolent autoimmune diseases.

Molecular diagnostics, including those based on gene expression, are increasingly being applied in the clinic (44, 45). These tests have improved the selection of therapies, as well as dosage and treatment schedule. In addition, “treat-to-normal” strategies are routinely used in major diseases such as hypertension and diabetes. Assays based on precise, quantitative measurements of immune system gene expression offer the promise of effective clinical monitors in infection, autoimmune diseases, other immune related conditions, such as transplant rejection and drug- or virus-induced immunosuppression, as well as cancer. A better understanding of the relevant factors that contribute to the individuality of gene expression in the human will help to establish the most appropriate normal reference values in the clinic and will serve as an essential

step in the development of effective molecular diagnostics for these and other inflammatory and immunologic diseases.

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# Figures and Tables

**Table 1: Genes with detectable expression in healthy blood donor samples, together with statistical summaries of $\Delta C_T$ distribution, expression fold changes corresponding to 2 standard deviations of $\Delta C_T$ distribution, and p-values for normality tests. N is the number of samples having detectable expression for the gene in at least 3 of 4 replicate RT-PCR reactions. Mean and SD are estimated by maximum likelihood for genes where any replicates fall below the detection threshold ($C_T$ > 37).**

| HUGO Designation | Gene Name and Aliases | N | Mean | Median | SD | Fold Change @ 2 SD | Shapiro-Wilk | Anderson-Darling |
|---|---|---|---|---|---|---|---|---|
| ADAM17 | A Disintegrin and Metalloproteinase Domain 17 | 129 | 18.56 | 18.55 | 0.63 | 2.39 | 0.7512 | 0.5395 |
| APAF1 | Apoptotic Protease Activating Factor 1 | 131 | 16.46 | 16.48 | 0.54 | 2.13 | 2.1E-05 | 0.0150 |
| C1QA | Complement Component 1, Q Subcomponent, Alpha Polypeptide | 128 | 20.25 | 20.21 | 0.92 | 3.57 | 0.0939 | 0.0879 |
| CD14 | CD14 Antigen | 129 | 13.92 | 14.01 | 0.63 | 2.41 | 1.1E-05 | 8.0E-07 |
| CD19 | CD19 Antigen | 131 | 18.19 | 18.09 | 0.78 | 2.94 | 1.4E-05 | 1.1E-07 |
| CD4 | CD4 Antigen | 131 | 14.80 | 14.84 | 0.49 | 1.98 | 0.0064 | 3.8E-04 |
| CD86 | CD86 Anitgen; B7-2 Protein | 128 | 17.64 | 17.68 | 0.51 | 2.04 | 3.1E-05 | 6.6E-04 |
| CD8A | CD8 Antigen, Alpha Polypeptide, p32 | 130 | 15.74 | 15.72 | 0.67 | 2.54 | 0.0653 | 0.8402 |
| CXCL1 | Chemokine (C-X-C Motif) Ligand 1 (GRO-1) | 131 | 20.01 | 20.00 | 0.67 | 2.53 | 0.1150 | 0.1522 |
| CYBB | Cytochrome B-245 Beta Polypeptide | 130 | 13.98 | 14.02 | 0.57 | 2.21 | 0.0058 | 0.0542 |
| DPP4 | Dipeptidylpeptidase IV (CD26) | 131 | 18.33 | 18.35 | 0.61 | 2.34 | 0.1253 | 0.0602 |
| EGR1 | Early Growth Response 1 | 130 | 20.42 | 20.49 | 0.65 | 2.47 | 0.0074 | 0.0013 |
| ELA2 | Elastase 2, Neutrophil | 126 | 19.90 | 19.78 | 1.29 | 5.95 | 2.1E-04 | 1.4E-04 |
| GCLC | Glutamate-Cysteine Ligase, Catalytic Subunit | 128 | 18.86 | 18.90 | 0.64 | 2.41 | 5.6E-07 | 2.9E-05 |
| HMGB1 | High-Mobility Group Box 1 | 130 | 16.28 | 16.25 | 0.69 | 2.59 | 0.0055 | 0.0524 |
| HMOX1 | Heme Oxygenase (Decycling) 1 | 131 | 16.45 | 16.50 | 0.67 | 2.53 | 0.0028 | 0.0045 |
| HSPA1A | Heat Shock Protein 1A, 70kD | 129 | 13.83 | 13.88 | 0.80 | 3.01 | 3.7E-08 | 1.2E-06 |
| ICAM1 | Intercellular Adhesion Molecule 1 | 131 | 17.68 | 17.71 | 0.55 | 2.15 | 0.0969 | 0.0514 |
| IFI16 | Interferon, Gamma-Inducible Protein 16 | 130 | 16.75 | 16.72 | 0.84 | 3.20 | 0.0441 | 0.1004 |
| IL10 | Interleukin 10 | 75 | 22.87 | 22.94 | 0.75 | 2.81 | 9.0E-04 | 0.0070 |
| IL15 | Interleukin 15 | 129 | 21.45 | 21.45 | 0.70 | 2.65 | 0.0051 | 0.0275 |
| IL18 | Interleukin 18 (Interferon Gamma-Inducing Factor) | 130 | 20.05 | 20.05 | 0.54 | 2.11 | 0.0517 | 0.0625 |
| IL18BP | IL-18 Binding Protein | 131 | 16.74 | 16.72 | 0.44 | 1.84 | 0.2787 | 0.6132 |
| IL1B | Interleukin 1, Beta | 130 | 16.67 | 16.67 | 0.79 | 2.99 | 0.0011 | 0.0200 |
| IL1R1 | Interleukin 1 Receptor, Type I | 125 | 21.08 | 21.06 | 0.98 | 3.90 | 0.5969 | 0.9508 |
| IL1RN | Interleukin 1 Receptor Antagonist | 129 | 16.88 | 16.91 | 0.67 | 2.54 | 0.1494 | 0.1755 |
| IL8 | Interleukin 8 | 97 | 21.01 | 20.86 | 1.46 | 7.53 | 0.0321 | 0.1185 |
| LTA | Lymphotoxin, Alpha | 114 | 20.05 | 19.99 | 0.65 | 2.45 | 3.4E-04 | 0.0014 |
| MMP9 | Matrix Metalloproteinase 9 | 129 | 15.91 | 16.01 | 1.16 | 4.97 | 3.8E-05 | 1.9E-06 |
| MNDA | Myeloid Cell Nuclear Differentiation Antigen | 130 | 12.54 | 12.51 | 0.64 | 2.44 | 0.1193 | 0.1563 |
| MPO | Myeloperoxidase | 131 | 21.20 | 21.22 | 0.77 | 2.92 | 0.7944 | 0.7479 |
| MYC | V-myc Avian Myelocytomatosis Viral Oncogene Homolog | 130 | 17.23 | 17.22 | 0.63 | 2.38 | 0.0685 | 0.0705 |
| NFKB1 | Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-Cells 1 (p105) | 131 | 17.38 | 17.41 | 0.57 | 2.20 | 0.0178 | 0.0076 |
| PLA2G7 | Phospholipase A2, Group VII | 126 | 19.36 | 19.36 | 0.69 | 2.60 | 0.1485 | 0.5492 |
| PLAUR | Plasminogen Activator, Urokinase Receptor | 131 | 15.12 | 15.15 | 0.58 | 2.25 | 0.0275 | 0.0190 |
| PTGS2 | Prostaglandin-Endoperoxide Synthase 2 (COX-2) | 126 | 16.72 | 16.75 | 0.61 | 2.33 | 0.0505 | 0.0309 |
| PTPRC | Protein Tyrosine Phosphatase Receptor, Type C (CD45) | 127 | 11.91 | 11.96 | 0.52 | 2.07 | 0.0410 | 0.0165 |
| SERPINA1 | Serine (or Cysteine) Proteinase Inhibitor, Clade A, Member 1 (Alpha 1 Anti-Trypsin) | 131 | 13.26 | 13.27 | 0.66 | 2.50 | 0.0100 | 0.0092 |
| SERPINE1 | Serine (or Cysteine) Proteinase Inhibitor, Clade E (Ovalbumin), Member 1 (Plasminogen Activator Inhibitor Type 1) | 101 | 22.38 | 22.44 | 0.89 | 3.43 | 0.0014 | 0.0015 |
| SERPING1 | Serine (or Cysteine) Proteinase Inhibitor, Clade G (C1 Inhibitor), Member 1 (Angioedema, Hereditary) | 130 | 19.20 | 19.29 | 1.20 | 5.25 | 3.2E-04 | 6.0E-05 |
| TGFB1 | Transforming Growth Factor, Beta 1 | 130 | 13.14 | 13.16 | 0.44 | 1.83 | 0.0115 | 0.0141 |
| TIMP1 | Tissue Inhibitor of Matrix Metalloproteinase 1 | 131 | 15.02 | 15.09 | 0.57 | 2.19 | 1.1E-05 | 4.2E-06 |
| TLR2 | Toll-Like Receptor 2 | 130 | 16.07 | 16.13 | 0.63 | 2.38 | 0.0058 | 0.0015 |
| TNF | Tumor Necrosis Factor | 124 | 20.67 | 20.55 | 0.98 | 3.90 | 1.1E-05 | 3.4E-04 |
| TNFSF5 | Tumor Necrosis Factor (Ligand) Superfamily, Member 5 (CD40 Ligand) | 131 | 17.69 | 17.67 | 0.63 | 2.38 | 1.5E-14 | 1.5E-10 |
| TNFSF6 | Tumor Necrosis Factor (Ligand) Superfamily, Member 6 (Fas Ligand) | 126 | 20.41 | 20.35 | 0.74 | 2.80 | 4.7E-04 | 0.0021 |

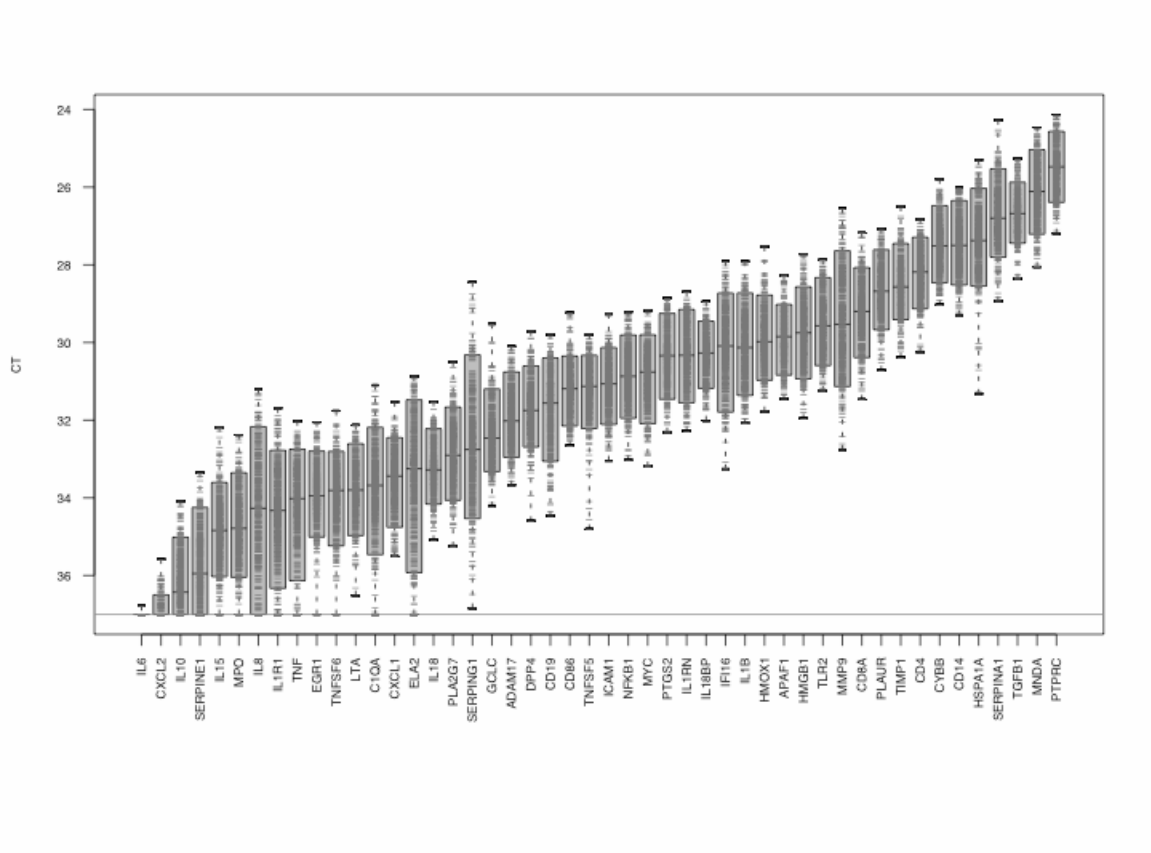



**Figure 1: Gene Distribution Across 131 Healthy Donors. Range of $C_T$ values for each gene targeted by the panel of 48 primer sets, across 131 single-time samples. Bars span the range from the 5$^{th}$ to the 95$^{th}$ percentile of $C_T$ values for each gene.**

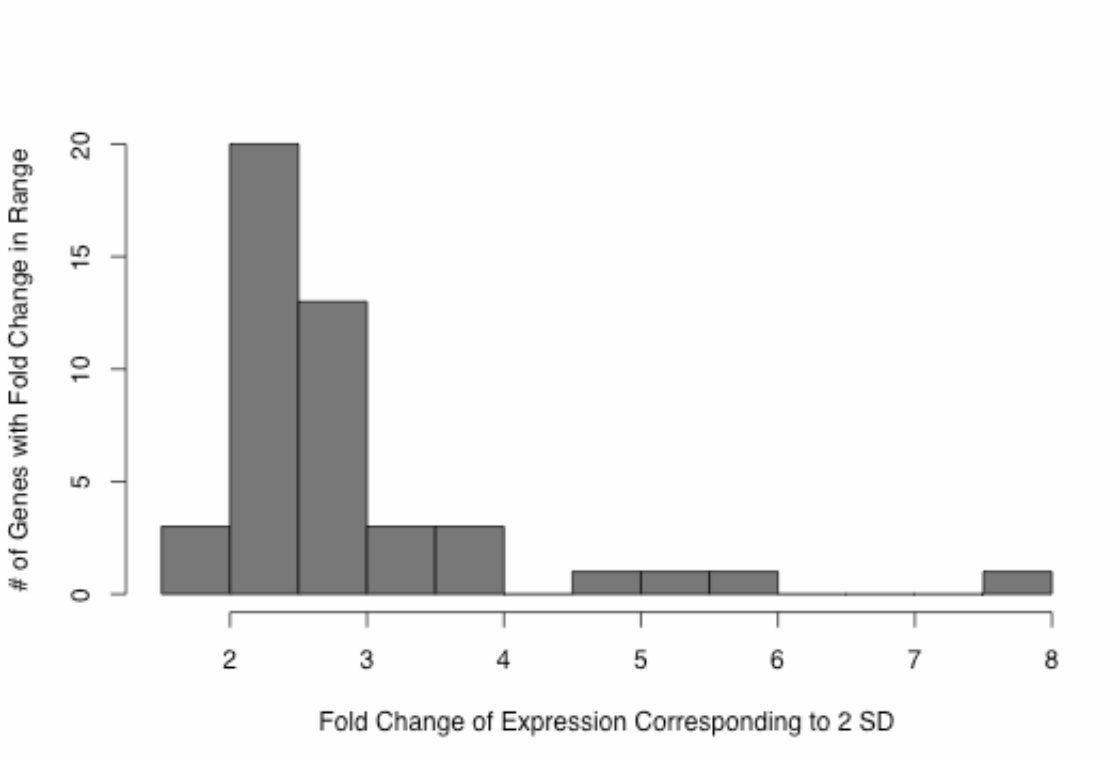
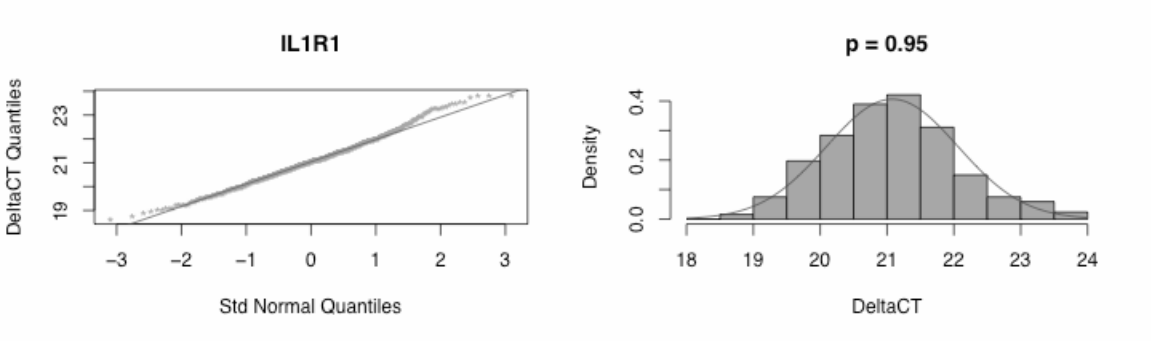



**Figure 2: Histogram of dynamic ranges of expression values, expressed as fold changes spanning two standard deviations of each gene's $\Delta C_T$ values (that is, $2^{-2SD(\Delta C_T)}$).**

A




B

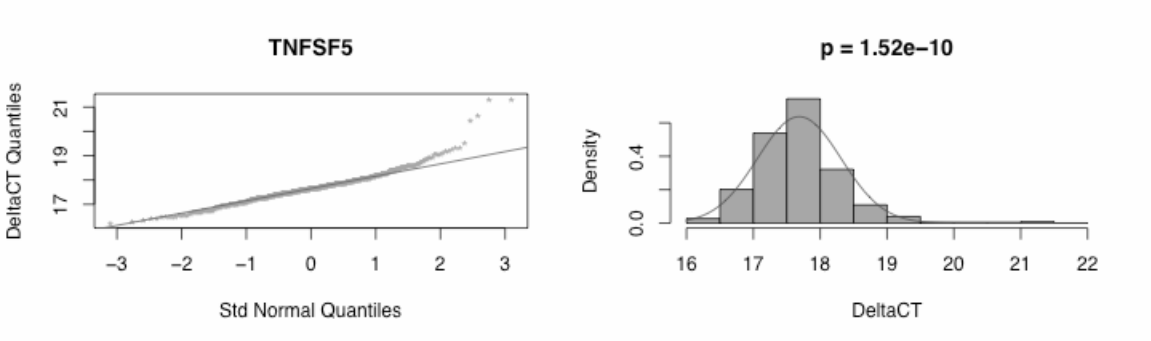
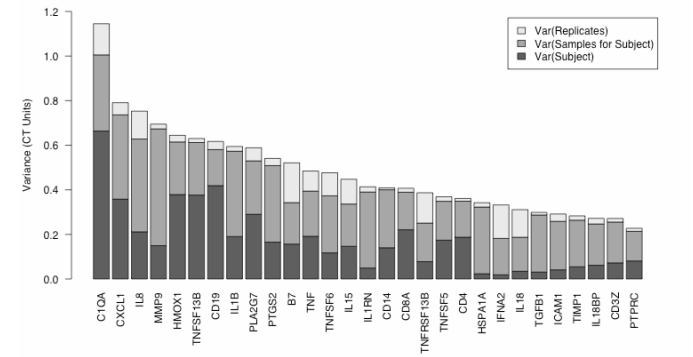



**Figure 3: Q-Q normal plots and histograms of $\Delta C_T$ values for the genes deviating least and most from a normal distribution (IL1R1 in Figure 3A and TNFSF5 in Figure 3B, respectively), according to the Anderson-Darling test. Unit diagonals and normal density curves are drawn on the Q-Q normal plots and histograms, respectively, for comparison with a normal distribution with the same mean and variance as observed. P-values computed by the Anderson-Darling normality test are shown above each histogram.**

**A**




**B**

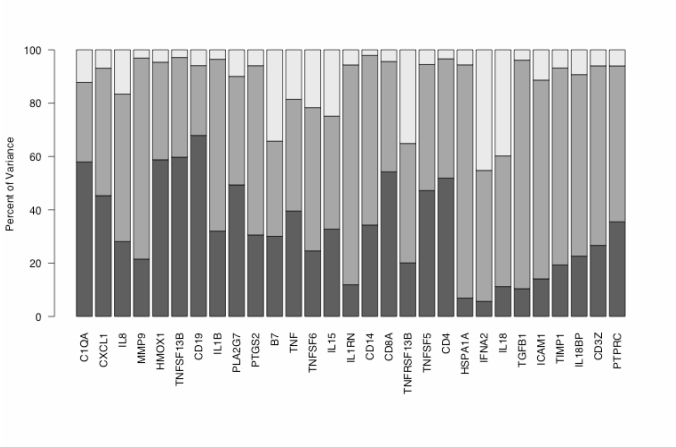



**Figure 4: Source of Variance in Gene Expression. (A) Variance components estimated from mixed-effect models, representing variation between subjects (dark grey), between longitudinal samples from same subject (grey), and between replicate RT-PCR reactions for same sample (white). Systematic variations affecting all samples drawn on same date have been subtracted before estimating variance components. (B) Variance components expressed as percentages relative to sum of components.**

**Table 2 : Gender, age and ethnicity (fixed effect) contributions to intersubject variation for 43 genes, in decreasing order of percentage of variance explained (equation 3). Values were computed only for white and Hispanic subjects for whom gender and age were recorded (N=68). Unadjusted p-values are shown for each effect, including interaction terms, and highlighted (together with corresponding fold changes) when < 0.05. Fold changes for gender and ethnicity effects are computed by raising 2 to the power of the corresponding $\Delta C_T$ effect terms; for age effects, they are computed by multiplying the corresponding slope effect by the range of ages in the sample (69 – 23) and then exponentiating.**

| Gene | % of Variance Explained by Gender, Age, and Ethnicity | p-Values for Effect | | | | | | | Fold Change Corresponding to Effect | | | Fold Change Corresponding to Age Difference (69 vs 23) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Gender | Ethnicity | Gender + Ethnicity | Age | Gender + Age | Ethnicity + Age | Gender + Ethnicity + Age | White Male vs White Fem | Hisp Fem vs White Fem | Hisp Male vs White Fem | White Fem | White Male | Hisp Fem | Hisp Male |
| NFKB1 | 27.90 | 0.0022 | 0.7083 | 0.1098 | 0.3335 | 0.0553 | 0.1896 | 0.2572 | 1.35 | -1.04 | -1.03 | -1.29 | -2.48 | 1.32 | 1.43 |
| MPO | 27.74 | 0.0128 | 0.0005 | 0.0116 | 0.2260 | 0.3535 | 0.8682 | 0.0909 | 1.42 | 1.82 | 1.31 | 1.57 | 1.00 | 1.43 | 4.48 |
| IL18 | 27.46 | 0.0220 | 0.6228 | 0.9333 | 0.0119 | 0.0468 | 0.9150 | 0.1023 | 1.25 | 1.06 | 1.30 | 1.97 | -1.02 | 1.88 | 2.77 |
| MYC | 26.91 | 0.0180 | 0.0132 | 0.7298 | 0.5240 | 0.1344 | 0.9658 | 0.1843 | 1.31 | -1.42 | -1.17 | -1.21 | -2.19 | -1.24 | 1.23 |
| TGFB1 | 25.88 | 0.0121 | 0.0726 | 0.5024 | 0.5696 | 0.0809 | 0.3451 | 0.1967 | 1.22 | -1.18 | -1.07 | -1.13 | -1.84 | 1.21 | 1.47 |
| TNFSF6 | 23.90 | 0.0008 | 0.0344 | 0.0123 | 0.1039 | 0.0892 | 0.8499 | 0.4898 | 1.62 | 1.39 | 1.17 | 1.78 | -1.28 | 1.61 | 1.32 |
| LTA | 23.87 | 0.0179 | 0.8223 | 0.0131 | 0.4992 | 0.1109 | 0.9253 | 0.4470 | 1.32 | -1.03 | -1.33 | -1.22 | -2.32 | -1.17 | -1.27 |
| ELA2 | 23.65 | 0.3536 | 0.0013 | 0.0998 | 0.0262 | 0.2949 | 0.1695 | 0.0889 | 1.24 | 2.53 | 1.50 | 4.21 | 1.78 | 1.09 | 6.94 |
| CD86 | 21.03 | 0.0289 | 0.2781 | 0.4782 | 0.1418 | 0.1509 | 0.3672 | 0.3403 | 1.24 | 1.13 | 1.23 | 1.47 | -1.11 | 2.12 | 2.42 |
| CD14 | 20.75 | 0.0066 | 0.9157 | 0.1150 | 0.1484 | 0.4972 | 0.0631 | 0.6869 | 1.42 | -1.02 | -1.05 | -1.65 | -2.24 | 1.64 | 1.71 |
| C1QA | 19.11 | 0.9650 | 0.2648 | 0.7856 | 0.0458 | 0.1489 | 0.0286 | 0.0015 | 1.01 | 1.25 | 1.15 | 2.51 | 1.07 | -1.89 | 9.27 |
| GCLC | 19.04 | 0.1281 | 0.0510 | 0.4470 | 0.1961 | 0.5864 | 0.4943 | 0.1936 | 1.19 | 1.31 | 1.33 | 1.51 | 1.21 | 2.10 | -1.66 |
| HSPA1A | 18.91 | 0.0208 | 0.3329 | 0.8448 | 0.2111 | 0.5560 | 0.1404 | 0.3747 | 1.37 | -1.17 | 1.12 | -1.58 | -2.09 | 1.46 | 2.47 |
| TNF | 18.22 | 0.0489 | 0.6451 | 0.2638 | 0.6774 | 0.8716 | 0.0660 | 0.4877 | 1.44 | -1.11 | -1.14 | -1.23 | -1.11 | 3.34 | 1.59 |
| HMGB1 | 17.72 | 0.0630 | 0.0047 | 0.1891 | 0.2164 | 0.3968 | 0.6109 | 0.9320 | 1.24 | 1.50 | 1.39 | 1.48 | 1.05 | 1.16 | -1.31 |
| CYBB | 17.34 | 0.0223 | 0.9489 | 0.3272 | 0.9352 | 0.2462 | 0.1217 | 0.5252 | 1.31 | -1.01 | 1.05 | 1.03 | -1.56 | 2.17 | 2.22 |
| SERPINA1 | 17.27 | 0.0730 | 0.2471 | 0.9965 | 0.2167 | 0.5933 | 0.0938 | 0.5365 | 1.28 | -1.21 | 1.06 | -1.59 | -2.06 | 1.66 | 2.28 |
| MMP9 | 16.14 | 0.1858 | 0.1057 | 0.3289 | 0.1730 | 0.6537 | 0.5372 | 0.3199 | 1.36 | -1.57 | 1.34 | -2.39 | -3.46 | -1.31 | 2.56 |
| CXCL1 | 15.91 | 0.1965 | 0.1305 | 0.5228 | 0.3722 | 0.4229 | 0.1961 | 0.4267 | 1.18 | -1.26 | 1.09 | -1.36 | -1.94 | 1.46 | 2.02 |
| EGR1 | 15.53 | 0.0263 | 0.3387 | 0.4572 | 0.2550 | 0.0909 | 0.6418 | 0.2217 | 1.33 | -1.15 | -1.04 | 1.48 | -1.44 | 1.16 | 1.54 |
| IL15 | 15.44 | 0.4922 | 0.0501 | 0.0862 | 0.3364 | 0.9222 | 0.3302 | 0.2744 | 1.10 | 1.37 | -1.03 | 1.42 | 1.36 | 2.45 | -1.15 |
| DPP4 | 15.00 | 0.2114 | 0.4354 | 0.4450 | 0.1223 | 0.6509 | 0.1781 | 0.5079 | 1.15 | -1.11 | -1.14 | -1.63 | -1.95 | 1.18 | -1.70 |
| CD4 | 14.86 | 0.0816 | 0.4197 | 0.3359 | 0.9445 | 0.0935 | 0.4285 | 0.4223 | 1.19 | -1.10 | -1.11 | 1.02 | -1.77 | 1.42 | 1.34 |
| HMOX1 | 14.81 | 0.0587 | 0.8654 | 0.6194 | 0.2167 | 0.0801 | 0.9441 | 0.0954 | 1.27 | -1.03 | 1.10 | 1.53 | -1.44 | 1.59 | 3.05 |
| PLA2G7 | 14.20 | 0.0865 | 0.9282 | 0.7051 | 0.4431 | 0.8269 | 0.0575 | 0.6794 | 1.27 | 1.02 | 1.17 | -1.34 | -1.49 | 2.31 | 3.07 |
| PLAUR | 13.46 | 0.2046 | 0.4947 | 0.9590 | 0.4582 | 0.2254 | 0.6593 | 0.1645 | 1.17 | -1.10 | 1.07 | -1.28 | -2.15 | -1.02 | 1.83 |
| TIMP1 | 13.33 | 0.0658 | 0.5106 | 0.2000 | 0.4669 | 0.1399 | 0.8392 | 0.3714 | 1.23 | -1.09 | -1.17 | 1.25 | -1.44 | 1.37 | 1.51 |
| CD8A | 13.23 | 0.0412 | 0.1604 | 0.8832 | 0.2939 | 0.7299 | 0.4394 | 0.8534 | 1.30 | 1.24 | 1.56 | -1.44 | -1.24 | 1.05 | 1.44 |
| ADAM17 | 12.71 | 0.1919 | 0.8616 | 0.6441 | 0.1633 | 0.2687 | 0.7874 | 0.7724 | 1.15 | 1.02 | 1.29 | 1.49 | -1.01 | 1.68 | 1.37 |
| PTPRC | 12.65 | 0.0279 | 0.7490 | 0.7072 | 0.5853 | 0.4100 | 0.2291 | 0.5759 | 1.24 | 1.04 | 1.20 | -1.15 | -1.53 | 1.41 | 1.53 |
| PTGS2 | 12.55 | 0.0630 | 0.9846 | 0.6788 | 0.0812 | 0.9289 | 0.1552 | 0.9724 | 1.28 | -1.00 | 1.15 | -1.87 | -1.79 | 1.17 | 1.18 |
| IL1RN | 12.41 | 0.3848 | 0.0842 | 0.1495 | 0.1451 | 0.6460 | 0.1061 | 0.8547 | -1.13 | -1.33 | -1.03 | -1.73 | -1.38 | 1.48 | 2.18 |
| ICAM1 | 11.99 | 0.2755 | 0.2793 | 0.7173 | 0.4375 | 0.4882 | 0.2839 | 0.3328 | 1.13 | -1.16 | 1.06 | -1.27 | -1.68 | 1.31 | 2.10 |
| APAF1 | 11.91 | 0.0852 | 0.8732 | 0.8466 | 0.8508 | 0.4636 | 0.0957 | 0.6159 | 1.20 | -1.02 | 1.13 | -1.05 | -1.38 | 1.96 | 1.06 |
| MNDA | 11.64 | 0.0662 | 0.9441 | 0.3044 | 0.5092 | 0.6863 | 0.3526 | 0.3483 | 1.24 | -1.01 | -1.03 | -1.23 | -1.46 | 1.28 | 2.29 |
| IL18BP | 10.94 | 0.1220 | 0.0913 | 0.1308 | 0.6556 | 0.3362 | 0.4393 | 0.4857 | 1.14 | 1.18 | 1.06 | 1.11 | -1.20 | 1.45 | 1.62 |
| SERPING1 | 10.70 | 0.4339 | 0.2313 | 0.9868 | 0.5224 | 0.9825 | 0.7008 | 0.2993 | -1.21 | -1.41 | -1.69 | -1.52 | -1.49 | -1.03 | 5.36 |
| IL1R1 | 10.49 | 0.1039 | 0.7632 | 0.9654 | 0.7602 | 0.3975 | 0.2078 | 0.9550 | 1.34 | -1.07 | 1.28 | -1.16 | -2.00 | 2.25 | 1.40 |
| IL1B | 10.09 | 0.6746 | 0.3584 | 0.4113 | 0.3254 | 0.7338 | 0.1561 | 0.9919 | -1.07 | -1.19 | 1.01 | -1.52 | -1.84 | 1.68 | 1.40 |
| TLR2 | 9.82 | 0.2787 | 0.6278 | 0.5153 | 0.7197 | 0.1332 | 0.2841 | 0.8863 | 1.15 | 1.08 | 1.06 | 1.14 | -1.77 | 2.05 | 1.16 |
| TNFSF5 | 9.48 | 0.3867 | 0.3648 | 0.1478 | 0.0599 | 0.4390 | 0.3545 | 0.6951 | 1.12 | 1.16 | -1.12 | -2.01 | -1.39 | -1.19 | -1.18 |
| IFI16 | 6.46 | 0.5860 | 0.6442 | 0.5085 | 0.2437 | 0.8924 | 0.4439 | 0.9634 | 1.09 | 1.09 | -1.02 | -1.62 | -1.74 | 1.00 | -1.02 |
| CD19 | 5.24 | 0.1827 | 0.4304 | 0.1925 | 0.3513 | 0.1437 | 0.4404 | 0.5098 | 1.23 | 1.16 | -1.03 | 1.48 | -1.51 | -1.11 | -1.25 |

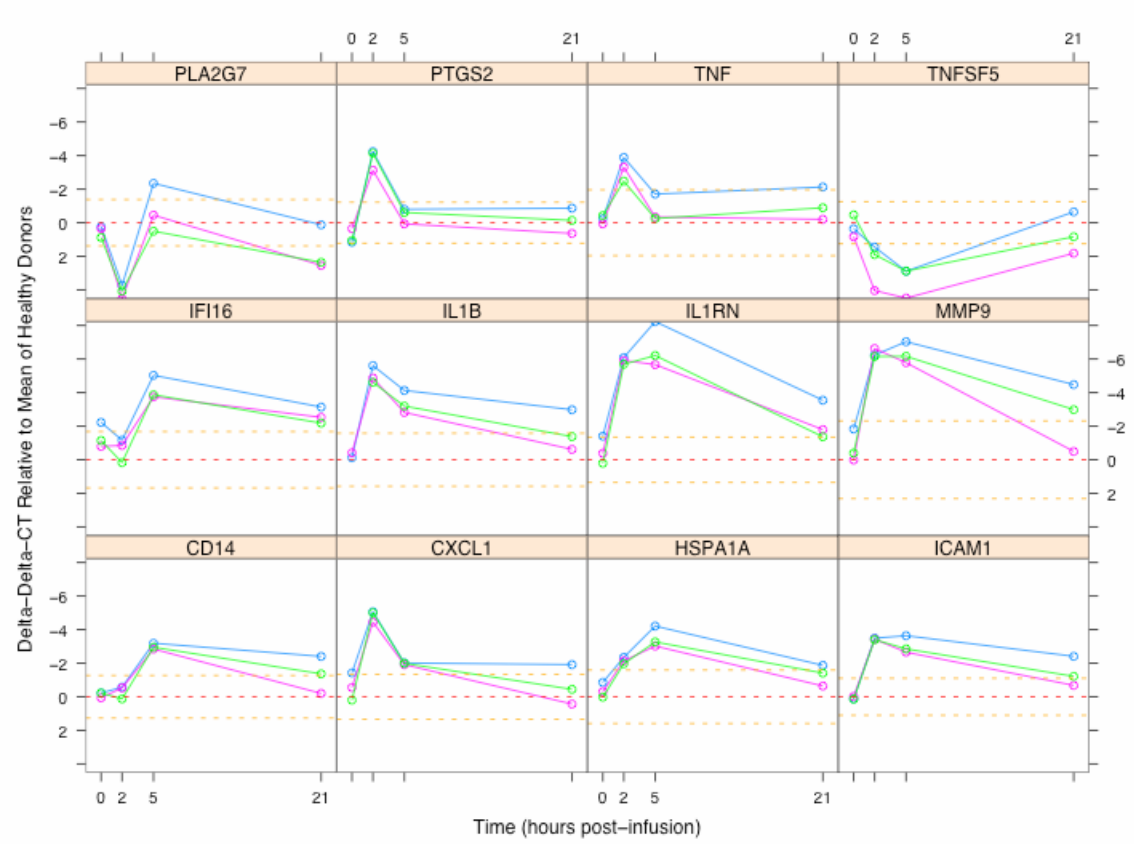



**Figure 5: Time course of expression for 12 genes with significant responses to LPS infusion in 3 healthy male subjects. Whole blood was sampled at pre-LPS (0h), 2, 5 and 21 hours post-LPS infusion. Gene expression is plotted as $\Delta C_T$ values relative to mean $\Delta C_T$ for healthy blood donors, with points and lines colored by subject. Mean and mean +/- 2 SD are indicated by horizontal dashed lines. $\Delta C_T$ scale is inverted, so that upward direction corresponds to increasing expression.**